ORIGINAL PAPER

The study of p53 and CA19-9 prognostic molecular markers in colorectal carcinomas

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Abstract

Immunohistochemistry represents a relatively new identification technique of cellular and tissular antigens due to an antigen–antibody binding interactions, applied more and more in the pathology laboratories for positive and differential diagnosis of premalignant and malignant lesions and also for evolutive prognosis of tumoral formations. Immunohistochemistry coloration is based on the antigen–antibody affinity, the antibody binding situs being identified either by antibody coloration direct methods or by using an indirect method where the marking is supplied by antibodies called secondary or even tertiary. The present study is based on the immunohistochemical investigation of some molecular markers with a prognosis value in colorectal cancers (CCR), like the antibody p53 immune expression and of some factors supposed to have prognosis value, such as CA19-9 (carcinoembryonic antigen, carbohydrate antigen or Lewis antigen). In the last decade, the studies have tried to define the prognosis of the molecular markers that allow the identification of the patients with recurrent risk after surgical treatment and who can benefit from chemotherapy in an efficient way. The purpose of the study on immunohistochemical markers is to aim towards the treatment based on molecular phenotypes of colorectal tumors. In 2000, according to the *Consensus Conference of the Colorectal Working Group of the American Joint Committee on Cancer Prognostic Factors* (AJC), there were established four categories of prognosis factors in colorectal cancers based on the practical importance and the number of studies referring to them. The present study is centered on the prognosis factors from the second B category (p53 oncoprotein expression) and the fourth category (CA19-9 immunomarker expression).

***Keywords*:** molecular markers in CCR, p53 antibody, CA19-9 antibody.

Introduction

According to the last world report in 2009 of the *Cancer Statistics* [1], colon cancer has an estimated incidence of 106 100 cases/year in both sexes (52 010 men and 54 090 women) and a number of 49 920 deaths/year in both sexes (25 240 men and 24 680 women). Globally, it is estimated that in both localizations (colon + rectum), cancer is on third place regarding incidence both in men and in women, with a percentage of 10% of the total number of cancers and also on third place both in men and in women regarding the mortality rate , with 9% of the total cancer deaths. There was established that there was a significant statistical growth of incidence rates for colorectal cancer in both sexes between 1983–2002, apart from the USA, where there was established a decrease of the incidence in both sexes [2]. On average, the survival rate of one, three and five years is approximately of 90%, 78%, respectively 40–70%, and the free interval of the disease is approximately of 68 months [3].

Colorectal cancer is a multi factor disease, involving various etiopathology, whether we refer to a family aggregation, or to an exposure to a series of environmental risk factors (including nutrition), other times being involved a series of inflammatory causes of the digestive tract or the association between these risk factors.

In the last decades, studies have tried to define the prognosis molecular markers that allow the identification of patients with a recurring risk after the surgical treatment and that may be eligible for effective chemotherapy treatment. Some of these markers may predict whether a tumor responds to a certain type of chemotherapy or it may predict the degree of the systemic toxicity for a certain type of treatment. In this direction, there was studied the individual expression of proteins and of some genes at the level of messenger RNA, by using PCR and immunohistochemistry techniques. The asserted purpose of some of these studies is to aim at the treatment based on molecular phenotypes of colorectal tumors in these patients [4].

From this point of view, our study was focused on the immunohistochemical investigation of some prognosis markers useful for therapy individualization in such patients.

Material and Methods

Our study was a retrospective one and it included 207 cases of colorectal carcinomas, selected from the patients hospitalized in the Clinics of Surgery within the County Emergency Hospital of Craiova, in 2008. The exeresis samples were sent to the Pathology Laboratory within the same Hospital for the histopathological exam. They were processed by the classic histopathological technique of fixation into 10% buffered formalin, paraffin embedding, microtome sectioning by obtaining sections of 3–5 μm and stained with Hematoxylin–Eosin and trichromic Masson. The histopathological diagnosis was performed in accordance with the *WHO criteria* (*WHO 2002 reference*). Consequently, for the immunohistochemical study we selected a number of 24 colorectal carcinomas, being representatives under the clinical and morphological aspect for the entire surveyed patients, using the Vector Laboratories technique (Catalogue No. PK-6200). Paraffined sections obtained on the histopathological examination were deparaffined and hydrated, and, afterwards, they were either or not antigen retrieval, the endogen peroxidase was blocked with 3% oxygenate water for 15 minutes, there had been performed the blocking non-specific sites with 2% bovine serum albumin (BSA) in PBS for 30 minutes, followed then by an incubation in a humidity chamber for one hour at room temperature with primary antibodies p53 and CA19-9. The main characteristics of primary antibodies are presented in Table 1. After that, there were employed the secondary antibodies and the Streptavidin–peroxide following the indications of Vecstain Universal Elite ABC kit, followed by the visualization of the reactions by means of 3,3'-diaminobenzidine (DAB) chromogene, while the nuclear counterstaining had been performed with Harris' Hematoxylin for 30 seconds.

In order to fulfill the objective proposed, there were investigated the markers shown below, in the Table 1.

Table 1 – ***Applied antibodies in the study of colorectal carcinomas***

Antibody	Clone	Producer / Code	Antigenic exposure Microwaves/Enzymatic digestion	Dilution	Applied technique
Monoclonal Mouse Anti-Human p53	DO-7	Dako / M7001	Seven cycles of 3 minutes on microwaves (750W) in Tris-EDTA pH 9	1:50 in PBS pH 7.2	ABC
Monoclonal Mouse Anti-Human CA 19-9	116-NS-19-9	Dako / M3517	Without exposure	1:50 in PBS pH 7.2	ABC

As external controls for p53 and CA19-9 were used mammary adenocarcinoma G3 samples as a control tissue.

We mention that for each antibody used, we created the positive external control – the negative external control using the same ABC/HRP technique.

From the 24 colorectal cancers selected from the patients recruited from the Emergency County Hospital of Craiova, 19 tumors were diagnosed in pT3 stage and five cases in pT4 stage, with remote (liver) metastases. The histopathologic examination highlighted the prevalence of adenocarcinomas cases without any mucinous composition, with various differentiation degrees (11), adenocarcinomas with mucinous composition (six), mucinous carcinomas (five) and carcinomas with various components (two) [5].

The external positive control was performed on normal tissues that are containing the investigated target antigen (positive sections). They were processed under the same circumstances as the investigated tumor. This test was the first one to be interpreted in order to verify the efficiency of used reactives and the precision of the applied technique.

In the case of the negative external control, instead of the primary antibody there was used specific immunoglobulin of the same isotopic kind, in the same dilution (first control level) as the primary antibody.

Due to the fact that in immunohistochemistry there is not any standardized or universally accepted method for the selection of histological fields for interpretation or for showing the results, within the interpretation and reporting of immunohistochemically processed cases, we applied the interpretation criteria proposed by Jasani B and Schmidt KW (1993), according to which the coloration intensity being estimated as follows:

- (+++), when coloration is intensely positive or specifically distributed "all over", clearly visible at low increase;
- (++), when coloration is focal or of moderate intensity, clearly visible only at median increase;
- (+), when coloration is weak or very focal, clearly visible only at an intense increase;
- (±), when coloration is very low, at the limit;
- (-), when coloration is negative.

There were excluded from this interpretation the fields that presented foldings of diagnosis sections, necrosis and hemorrhages.

The main clinical and epidemiological data and the results of the histopathological examinations are shortly given in Table 2.

Table 2 – ***Clinical and epidemiological data and the results of the histopathological examinations***

Clinico-morphological parameters	No of cases (n=207)	Percent
Age [years]		
<30	0	0%
30–50	18	8.69%
>50	189	91.30%
Sex		
M	127	61.35%
F	80	38.64%
Lesional topography		
Wright colon	39	18.84%
Left colon	51	24.63%
Transverse colon	10	4.83%
Rectosigmoidian junction	21	10.14%
Rectum	86	41.54%
Clinical stage		
I+II	42	20.20%
III	104	50.24%

Clinico-morphological parameters	No of cases (n=207)	Percent
IV	61	29.46%
WHO histopathological subtype		
Adenocarcinoma	182	87.92%
Mucinous carcinoma	9	4.34%
Sealed-ring carcinoma	1	0.48%
Undifferentiated carcinoma	1	4.48%
Adenocarcinoma + mucinous carcinoma	10	4.83%
Rare forms	4	1.93%
Differentiation degree		
Good	69	33.33%
Moderate	108	52.17%
Weak	27	13.04%
Undifferentiated	3	1.44%
TOTAL	*207*	*100%*

From the data presented in the table above, we can observe that the most frequent colorectal cancer expanded to person's age over 50, especially in males and located in the rectum and sigmoid. The clinical stadialization showed to us the high incidence of cases in advanced stages of illness, respectively stages III with 104 patients and IV with 61 patients. The metastases were mostly present in a hepatic localization.

Histopathologically there predominated the forms of adenocarcinoma with 182 cases (Figures 1–3).

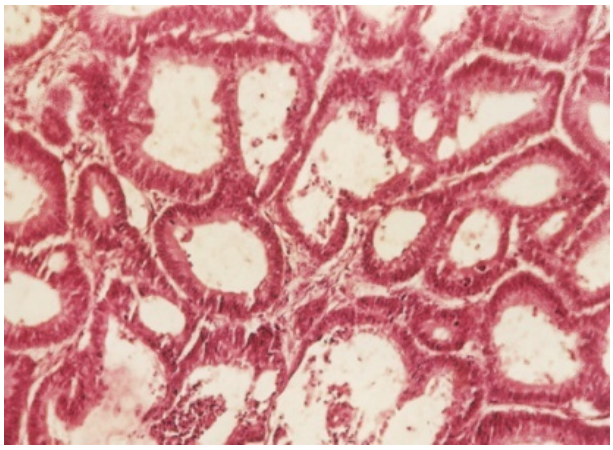

Figure 1 – ***Well-differentiated adenocarcinoma (HE stain, ×400).***

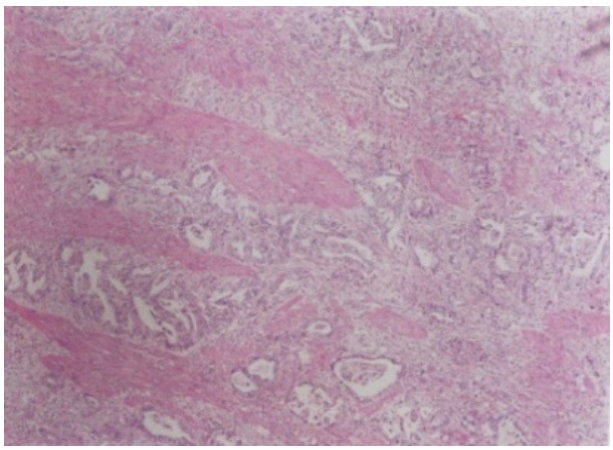

Figure 2 – ***Moderately differentiated adenocarcinoma (HE stain, ×400).***

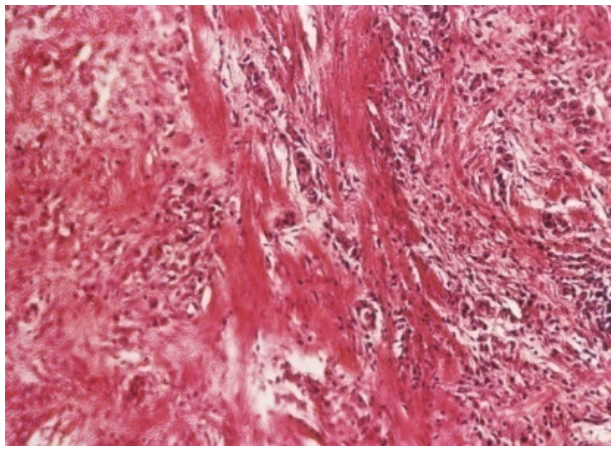

Figure 3 – ***Less differentiate adenocarcinoma with muscular tunic invasion (HE stain, ×100).***

On the second place regarding frequency, there were the mucinous forms with nine cases (Figures 4 and 5). On the last place, there were the undifferentiated carcinomas with one case. In 48 cases, there was encountered the presence of histopathological associations, mostly adenocarcinomas, 10 cases with mucinous carcinomas.

From the point of view of the differentiation degree, we noticed mostly the presence of moderate and well-differentiated forms, with 108 cases and 69 cases, respectively.

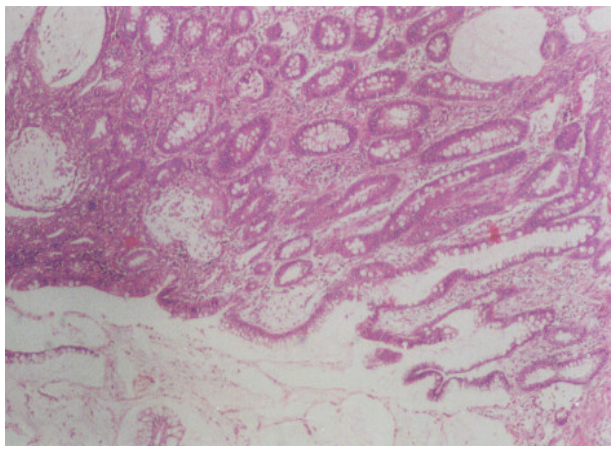

Figure 4 – ***Mucin-producing adenocarcinoma (HE stain, ×100).***

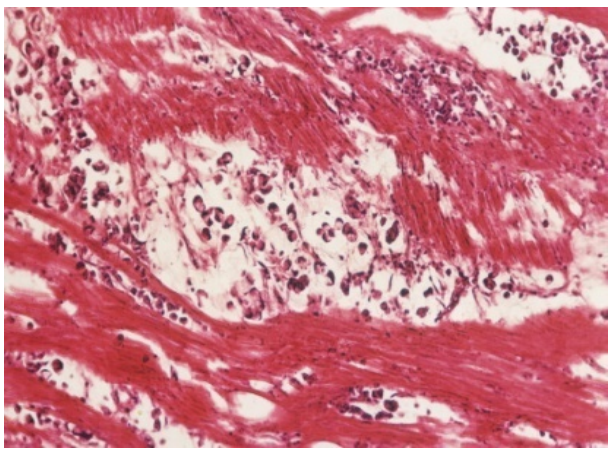

Figure 5 – ***Mucin-producing carcinoma with muscular tunic invasion (HE stain, ×400).***

The results of p53 antibody immunoexpression are presented in Table 3.

Table 3 – ***The quantitative evaluation of the immunomarker with anti-p53 antibody***

Adenocarcinoma varieties (No. of cases)	The score of qualitative evaluation of anti-p53 immunomarker				
	0	±	+	++	+++
Tubular-papillary (14)	1	–	2	4	7
Mucinous (6)	1	2	3	–	–
"Signet ring" (1)	–	–	1	–	–
Mixed (with more components) (3)	–	–	2	1	–

Immunoreactions were limited only to the epithelial component of the tumor. There not observed any immunoreactivity differences regarding the epithelial component based on the stroma / parenchyma ratio.

The immunoreaction was present in 22 of the investigated cases, representing 92% of the investigated patients. In the two cases with a negative reaction, there were involved weakly differentiated adenocarcinomas.

Related to the histopathologic subtype the lowest level of the p53 expression was registered in mucinous carcinomas. Practically, 20 investigated cases had a qualitative positive type reaction, and in two cases reactivity was at the limit. In two cases, we did not notice any positive reaction to this marker.

The maximum of intensity was obtained in the well-differentiated cases of tubulopapillary adenocarcinoma, respectively seven cases having a +++ intensity. In five cases the reaction was of moderate intensity (++), weak (+) in eight cases, at the limit (±) in two cases and negative in two cases.

Generally, we noticed a decrease of the immunoreactivity in colon adenocarcinomas with the decrease of the differentiation degree. The highest expression rate was established in well-differentiated adenocarcinomas (Figure 6), while the lowest rate was obtained in the case of weakly differentiated adenocarcinomas.

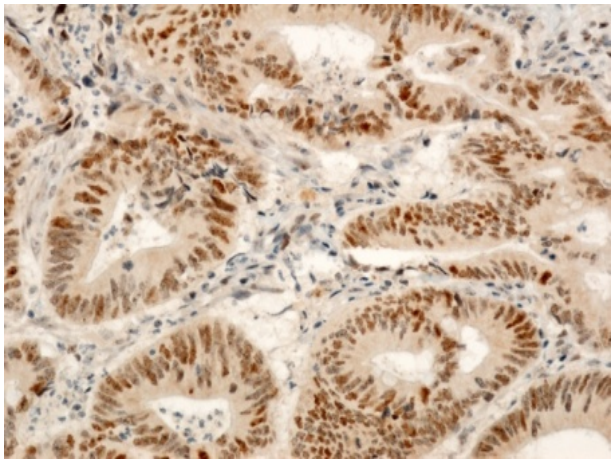

Figure 6 – ***Well-differentiated adenocarcinoma with intense reaction to p53 (×200).***

The histopathological mucinous and mixed subtypes had an intermediary reactivity regarding the other types of adenocarcinoma. Most of them had a moderate reaction (++) to this marker (Figure 7).

Moreover, we noticed the fact that the p53-expression was inversely proportional correlated to the gravity of tumoral invasion (Figure 8).

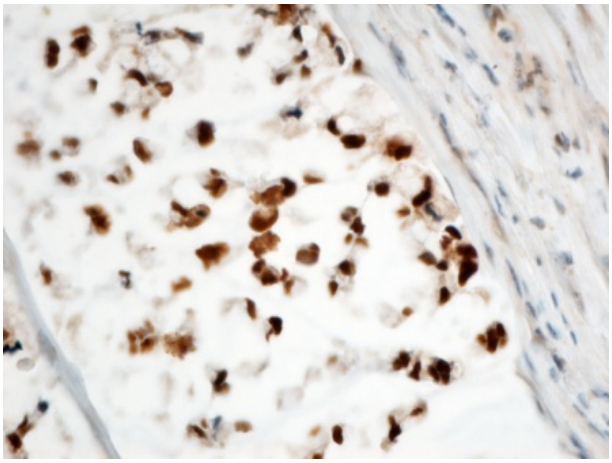

Figure 7 – ***Mucinous adenocarcinoma with weak reaction to p53 (×400).***

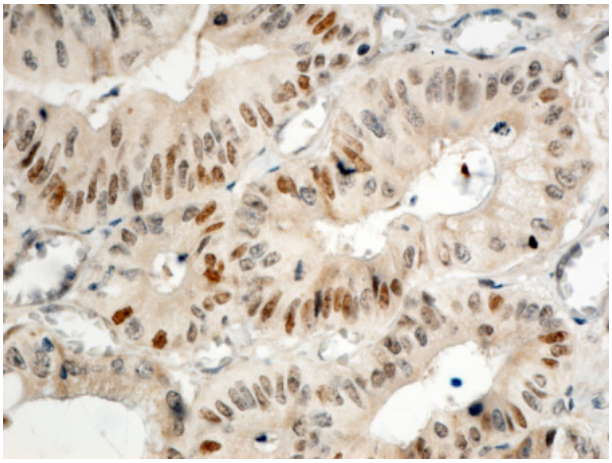

Figure 8 – ***Well-differentiated adenocarcinoma stage pT3 with weak reaction to p53 (×400).***

The results of immunohistochemical investigation of the casuistic for the antibody anti-CA19–9 are shown below, in Table 4.

Table 4 – ***The qualitative evaluation of immunomarker with anti-CA19-9 antibody***

Adenocarcinoma varieties (No. of cases)	The score of qualitative evaluation of anti-CA19-9 immunomarker				
	0	±	+	++	+++
Tubular-papillary (14)	7	1	1	2	3
Mucinous (6)	5	–	1	–	–
"Signet ring" (1)	–	–	1	–	–
Mixed (with more components) (3)	2	1	–	–	–

At normal tissues, the expression of this marker was limited only for the cells from the crypts of Liberkuhn's glands, the pattern of the expression being an apical membranous and cytoplasmatic supranuclear one.

Immunoreaction was present in 10 of investigated cases, representing 41.6% of the investigated casuistic. The 14 negative cases histologically corresponded to four cases of weakly differentiated tubulopapillary adenocarcinomas, three cases of moderately differentiated

tubulopapillary adenocarcinomas, five cases of mucinous adenocarcinomas and two cases of mixed adenocarcinomas.

Out of the 10 cases with positive immunoreactions, there was a variable (±) reaction in two cases, weakly (+) positive reaction in three cases, a moderate (++) positive reaction in two cases and an intensely (+++) positive reaction in three cases.

The highest intensity of the immunoreaction was observed in the forms of the well-differentiated adenocarcinoma, the reaction pattern being an apical membranous, cytoplasmatic and secreted one in the visible lumen of neoplasic glands (Figure 9).

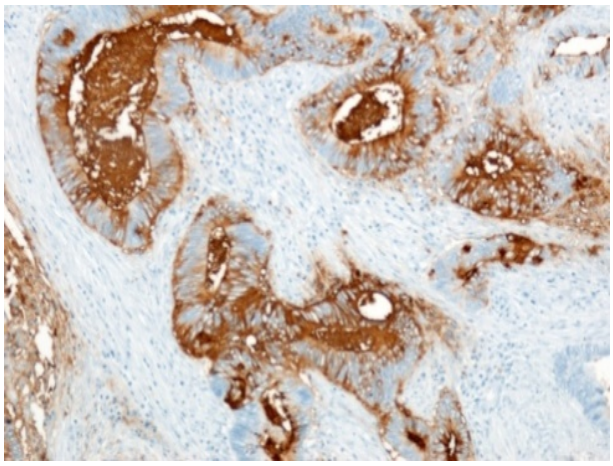

Figure 9 – ***Well-differentiated adenocarcinoma with intense reaction at CA19-9 (×100).***

The moderate intensity of this reaction was present in moderately and weakly differentiated forms; the immunostaining pattern was mainly a membranous one (Figure 10).

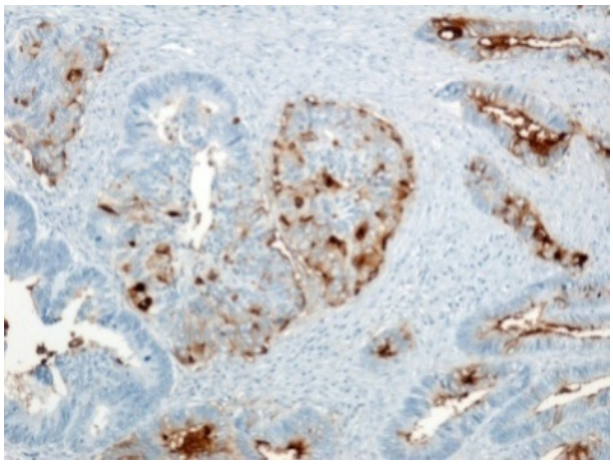

Figure 10 – ***Moderate differentiated adenocarcinoma with moderate-intense reaction at CA19-9, predominant membranous apical (×100).***

In weakly differentiated forms there were also distinguished a cytoplasmatic staining (Figure 11).

Even if generally the immunomarking in the mucinous forms was one of weak intensity, the reaction pattern was heterogeneous, within the same tumor being identified areas with intense marking (Figure 12), together with tumoral areas of moderate and weak intensity and even an absence of the marking.

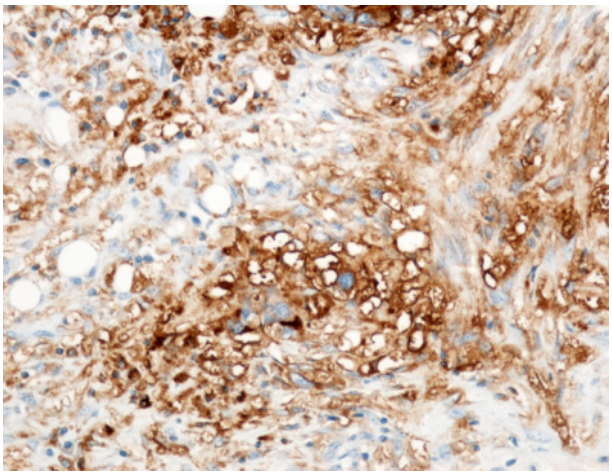

Figure 11 – ***Weak-differentiated adenocarcinoma with moderate reaction at CA19-9, membranous and cytoplasmatic apical marker (×200).***

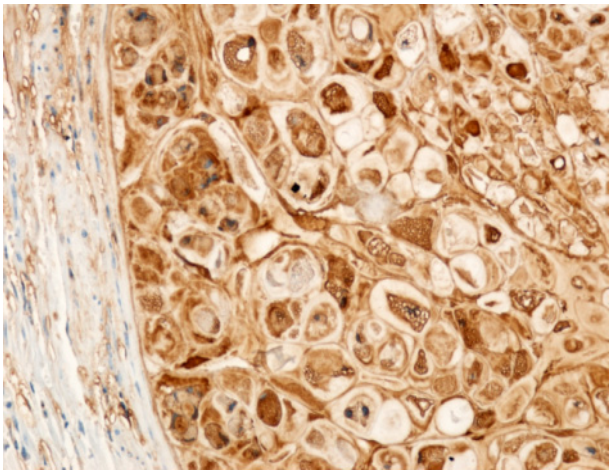

Figure 12 – ***Mucinous adenocarcinoma with intense reaction at CA19-9, membranous and cytoplasmatic apical marker (×200).***

In the mixed forms, the marking was more obvious at the level of the areas with adenocarcinoma differentiation, tumoral mucinous areas having a weak intensity or even absent at CA19-9 (Figure 13).

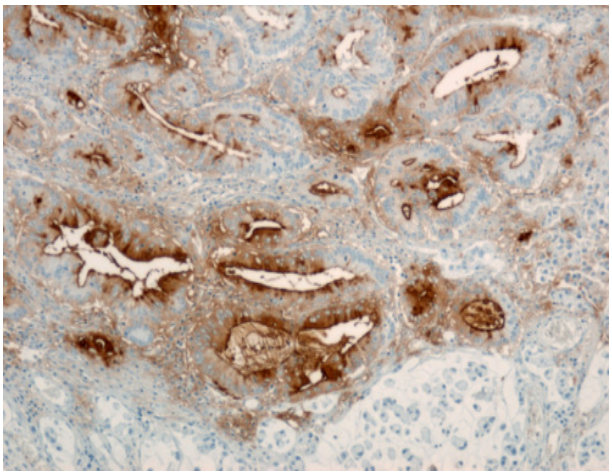

Figure 13 – ***Mixed adenocarcinoma, where the marker at CA19-9 is obvious at area's level of well and moderate differentiated and weak and even absent adenocarcinoma in the area of mucinous adenocarcinoma (×100).***

In the invasive forms of adenocarcinoma, the intensity of the staining in the invasion areas varied, the reaction having a luminal prevalence (Figure 14).

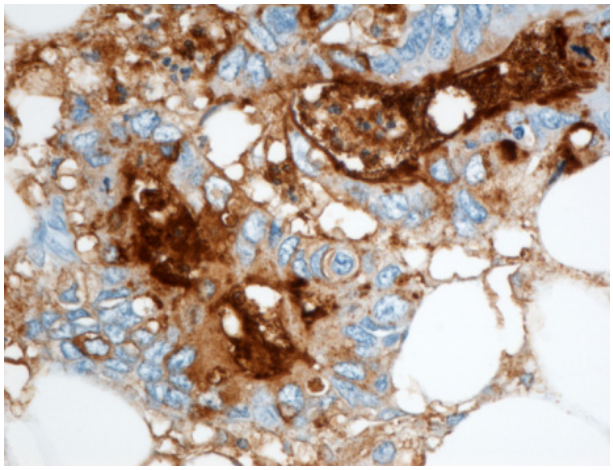

Figure 14 – ***Moderate differentiated adenocarcinoma with moderate intensity at CA19-9, prevail membranous apical and luminal (×200).***

Discussion

The epidemiologic profile of the patients with colorectal cancer patients hospitalized in the Clinics of Surgery within the Emergency County Hospital of Craiova, in 2008, show the prevalence of this type of cancer especially at the age over 50 years in men, having a sigmoid and rectum localization.

In 1996, generally, there was estimated a number of 875 000 cases of colorectal cancer, which represented approximately 8.5% of the total number of new cancer cases estimated [6]. The standardized incidence on the age groups varied a lot world widely, with geographic differences up to over 20 times, the highest rate being registered in the population from Europe, North and South America, Australia / New Zealand and from the developed countries and poorly developed countries like Malaysia, Korea in Asia, African countries and poorly developed states from Asia and Polynesia.

In the USA, according to the data of the *National Institute of Cancer Study* for 2009 there was stipulated a number of 106 100 new cases of colon cancer (52 010 in men and 54 090 in women) out of which 49 920 were to die (23 540 men and 24 680 women), while with a rectum localization there had been stipulated a number of 40 870 new cases (23 580 men and 17 290 women) [1, 2]. Despite the general tendency of the decrease in the incidence of colorectal cancer in USA, between 1992 and 2005 [7], there was estimated that there occurred an increase of this cancer incidence in persons aged below 50 years old, an increase of 1.5%/year in men and 1.6%/year in women.

Topographically speaking, in the USA [8], after a retrospective study (1980–1999), some authors observed an increase in the number of cases at the level of the right colon, from 46 to 58%, while [9] other authors give for the same localization a growth from 31 to 44% between 1978 and 1988.

The clinical diagnosis of the investigated patients showed that the diagnosis of colorectal cancer was performed in the advanced stages, respectively stages III and IV in an extent of 79.7%. The metastases were presented in 19% and were mainly localized in the liver in 10% of cases.

In a study [10] regarding a casuistic of 146 cases of colorectal cancers, pointed out that the greatest part of patients was diagnosed in advanced stages of the disease, 64.4% being in stage III. Another study [11] reported the following proportions regarding the TNM classification of colorectal cancers: stage I 7%, stage II 18%, stage II 57%, and stage IV 17%.

Literature data are revealing the importance of lymphatic ganglions metastases as independent prognosis factors in colorectal cancers [12, 13], this thing being especially present in recto-sigmoidian cancers [14].

Histopathologically, our study distinguished the high incidence of adenocarcinoma types. Other types were less represented in our casuistic. From the differentiation degree point of view, moderately differentiated forms were prevalent (108 cases). Nonetheless, we observed an important number of well-differentiated forms in 69 cases.

Literature data indicate the fact that adenocarcinoma represents over 95% of colorectal cancers [15] and there normally prevail the types with differentiation degrees G1 and G2. The mucinous carcinomas with a more unfavorable prognosis in comparison to adenocarcinomas represent the second histopathological type regarding frequency in colorectal cancerous pathology, literature data indicating variable percentages, from 3.62% [16], to 11.67% [17] and 30.5% [18].

Our study especially focused on the prognosis factors in the second B category (the expression of p53 oncoprotein) and on some factors supposed to have a prognosis value (CA19-9).

The mutations of p53 oncogene, suspensor tumoral gene, located on p17 chromosome are amongst most frequent genetic alterations from human cancers. Consequently, the anomalies of p53 gene have intensely been investigated in the last two decades, including their importance for the prognosis and for the response to therapy in colorectal carcinomas. From this perspective, the obtained results are contradictory ones [19–23], on the one side half because the anomalies of p53 gene are investigated through various methods which, normally, do not aim at the functional status of the two alleles of this gene.

The investigation of p53 marker expression in our casuistic pointed out a positive of the reaction in 92% of the cases, the intensity of the immunostaining decreesing at the same time with the decrease of the differentiation degree. Related to the histopathological subtype, the lowest level of p53-expression was registered in mucinous carcinomas, and, in addition, at the level of p53-expression it was correlated inversely proportional to gravity of the tumoral invasion.

The review on the data from literature about anomalies of p53-gene in colorectal cancers (168 paper works with a worldwide number of 18 766 investigated cases) distinguished the existence of a huge variety in the methodology of the determination of p53 status and

of the survival rate appreciation based on this one in patients with investigated colorectal cancers [22].

Reviewing the data regarding p53 as a prognosis potential marker in colorectal cancers there was observed that the immunohistochemical abnormal p53-expression of and the p53-gene mutation are both associated with a decreased risk of mortality. The adverse effect of p53-gene was higher in patients with a good prognosis, appreciating that for every 10% increase in the mean survival rate, the absolute rate of the difference of association with p53-anomalies decreases by 6%.

Also, there was recorded the fact that the p53-gene mutations increase the risk of metastases development, but immunohistochemistry cannot provide such observations. Even if p53 anomalies were more frequent in the rectum localization of carcinomas, the adverse effects of these mutations had the same impact for both localizations.

By reviewing the data regarding the function of the p53 predictive marker, there was noticed that p53-mutations were associated with failure of radiotherapy and chemotherapy in cases of rectal carcinomas, but without any predictive role played by immunohistochemistry.

Immunohistochemical studies regarding the CA19-9 antigen expression at the gastrointestinal tract level are quite few and not very recent ones. Hereby, Bara J *et al.* pointed out the CA19-9 expression in the fetal colon mucosa and in colorectal cancers, but only occasionally in normal adults mucosa [24]. A series of studies demonstrated an increased expression of this marker in the mucosa adjacent to colorectal adenocarcinoma [25–28]. Colonic premalignant tissues, like adenomatous polyps [27, 29], and ulcerous colitis [30] with dysplasia are also expressing this marker.

In our study, the percent of positive colorectal adenocarcinomas for this marker increased to 42%. The most intense immunostaining was in well-differentiated adenocarcinoma forms and the minimum level of expression was noticed in weak forms. An intermediary situation was that of mucinous forms, which presented a heterogeneous immunoreaction. The immunostaining pattern was predominantly apical membranous and luminal, but in weakly-differentiated forms we also observed the cytoplasmatic expression of this marker.

In the study conducted by Itzkowitz SH *et al.* [31] there was observed an overexpression of CA19-9 in 2/3 of cases of the investigated hyperplasic polyps, adenomatous polyps and transitional colonic mucosa, but not in the fetal or adult colonic mucosa. Generally, the expression of this marker was recorded in 70% of the hyperplasic polyps, in 100% of the adenomatous polyps, in 46% of the cases with transitional colonic mucosa and in 90% of the cases with investigated colon cancers [31]. Similarly to our results, the authors have shown that the CA19-9 antigen was intensely expressed at membrane level and in glands lumen and very little at the cytoplasm of cancerous cells.

In the study conducted by Shimono R *et al.* [32] at 149 patients with primary colorectal carcinomas there was observed a positive immunostaining for CA19-9 in 56% of the investigated tumors. The authors distinguished the existence of three patterns of immunoreactivity:

- first type with stromal reactivity present in 22 cases;
- second type with apical and/or cytoplasmatic reactivity present in 64 cases;
- third type without any reactivity present in 63 cases.

Moreover, the authors recorded the fact that the survival rate of 5 years for the patients in the first group of immunoreactivity to this marker was significantly increased in patients with more than three lymph nodes tumoral invaded. The authors concluded that the cytosolic level of CA19-9 at patients with colorectal carcinomas is an independent prognostic factor of recurrences. Hereby, they showed that at every growth of 5000 U/mg of tumoral cytosolic level the recurrences risk for the patients with colon carcinomas is 4, two times bigger while those with rectal cancers this risk is growing over 9.5 times [33].

A series of studies showed that the CA19-9 antigen is secreted in serum and the level of its secretion may be used as a tumoral marker in pancreatic, hepatobiliary carcinomas, from the gynecological area and in the colorectal one, as well [34–37]. Nevertheless, while some studies have shown the importance of measuring the serum level [38–47], others have not succeed in proving the utility of this intercession [48–52] and, consequently, this was not recommended for being included in the prognosis handbooks [35, 53, 54].

For the time being, the true importance of the serum level determination of CA19-9 is limited only to the appreciation of inoperable colorectal carcinomas prognosis, in the IV-th stage [40].

Conclusions

Our study pointed out a great sensibility of the colorectal adenocarcinomas to the p53 immunostaining, its expression being correlated with the tumoral differentiation degree, with the tumoral invasiveness degree and with the risk of metastases development, thus representing a certain factor for the progression and prognosis of colorectal cancers. CA19-9 antigen, having a smaller sensibility and specificity, the marker did not constitute a certain prognosis factor for colorectal carcinomas, but our investigation distinguished the possibility of its use as a marker for tumoral recurrences.

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